Interaction between polyether- poly (2-dimethylaminoethyl methacrylate) copolymers and phospholipid model vesicles – implications for gene delivery efficiency

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ABSTRACT

Our previous studies have demonstrated that surface-active copolymers of Pluronic® surfactants and tertiary amino polycations such as poly (2-dimethylaminoethyl methacrylate) (PDMA) possess an enhanced DNA transfection efficiency compared to PDMA homopolymers. In order to understand the mechanism of such action, we conducted model studies of the interaction of polymer with phospholipid vesicles and cell membranes. Three cationic methacrylate polymer systems were used in this study: PDMA homopolymer, and PEO-*b*-PDMA and Pluronic L92-*b*-PDMA block copolymers. The results showed that PDMA and PEO-*b*-PDMA only interact with the surface of the vesicles, whereas L92-*b*-PDMA interacts strongly with the inner hydrophobic segment of the vesicles. The studies show that the strong interaction between L92-*b*-PDMA and phospholipid membrane could be used to explain the enhanced transfection efficiency previously shown by the Pluronic L92-*b*-PDMA copolymer.

Keywords: fluorescence anisotropy, pluronic copolymers, poly (2-dimethylaminoethyl methacrylate), carboxyfluorescein (CF) release assay, phospholipids vesicles

1 INTRODUCTION

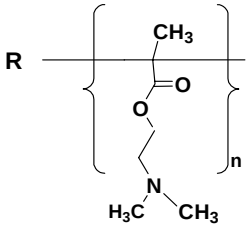
Non-viral polymeric gene delivery systems have become an attractive route as viral delivery systems pose safety concerns yet to be resolved [1]. Great efforts have been undertaken to improve the efficiency of the polycations however, satisfactorily systems have yet to be achieved primarily due to poor understanding of the cellular transfection barriers [2]. Cationic poly(dimethylamino)ethyl methacrylate (PDMA) systems is one of the systems that received considerable attention from the research field of gene delivery vector [3, 4]. Previously, our group has coupled PDMA with Pluronic L92 polymer which provide greater cell transfection efficiency compared to PEO-*b*-PDMA. Addition of Pluronic P123 further increased the transfection efficacy of L92-*b*-PDMA, but did not affect that of PEO-*b*-PDMA [3].

Understanding the intra-cellular trafficking is of utmost importance for the design of a efficient gene transfection carrier. However, many of the intra-cellular transfection barriers are poorly studied and understood [2]. Recent studies have shown that interaction between the cellular membranes and PDMA polyplexes is one of the main barriers during transfection [5, 6]. In this study, we investigated the molecular interaction between the various PDMA systems (namely PDMA homopolymer, PEO-*b*-PDMA and L92-*b*-PDMA copolymer) with phospholipids model bilayer membrane using different fluorescence techniques: Carboxyfluorescein (CF) release assay and fluorescence anisotropy study. The results showed distinctly different interaction mechanisms between the various PDMA systems and phospholipids vesicles. L92-*b*-PDMA demonstrated extensive interaction with the phospholipids vesicles, while PDMA and PEO-*b*-PDMA have minimum interaction.

2 MATERIALS & METHODS

Materials. 1,6-Diphenyl-1,3,5-hexatriene (DPH), 5(6)-Carboxyfluorescein (CF), 1,2-Dipalmitoyl-glycero-3-phosphocholine (DPPC) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) . Pluronic L92 (average composition, $EO_8PO_{52}EO_8$) was received from BASF Corp. (Mount Olive, NJ).

Polymer Synthesis. Polymerization of the L92-*b*-PDMA is carried out with a cerium ion redox system, full description of the synthesis has been reported [3]. Synthesis of PEO-*b*-PDMA and PDMA is by atom transfer radical polymerization (ATRP), details has been reported [7]. The degree of polymerization (DP) for L92-*b*-PDMA is about 23 repeat units and for the PEO-*b*-PDMA and PDMA are about 65 -75 repeat units of methacrylate monomers which was determined vs GPC and ^{1}H-NMR (Figure 1). The nominal number-average molecular weight of PEO (Dow Chemical) used is of $M_n \sim 5000$.



R =	OCH3	-	PDMA
	PEO	-	PEO-*b*-PDMA
	L92	-	L92-*b*-PDMA

Figure 1: Chemical structures of polymer systems used in this study.

Liposome Preparation. Liposomes were prepared using DPPC as follows. Solution of DPPC in chloroform (1mM) was placed in a round-bottom flask and the solvent was evaporated under vacuum at 55 ^{o}C. The resulting film was dried under vacuum for 2 h and then hydrated with 50 mM Tris-HCl buffer (pH 7.4). The solution is further underwent three freeze-thaw cycles using liquid nitrogen. Small vesicles were then obtained through further sonication in an ultrasounds bath above 50 ^{o}C for 1 h. Weight-average diameter of the vesicles (about 180nm) was measured using dynamic light scattering (Brookhaven Instrument Co.).

Measurement of fluorescence anisotropy. Anisotropy of DPH was applied to monitor changes in membrane dynamics [8, 9]. To load liposome solutions with DPH, 0.05 mL of 0.2 mM DPH solution in methanol was added to the stock liposome solution to a final DPH concentration of 1 μM and the resulting mixture was stirred in the dark at 55 ^{0}C for 60 min to reach equilibrium. Emission spectra were recorded between 410 and 430 nm (λ_{ex} = 360 nm), with a band-pass of 8 nm and integration time of 5 s. Polarized steady fluorescence intensity were recorded on a TimeMaster Fluorescence Lifetime Spectrometer (Photon Technology International, Canada) using a FeliX software for data acquisition. The steady-state fluorescence anisotropy <*r*> was calculated as [10]:

$$<r> = (I_{vv} - gI_{vh})/(I_{vv} + 2gI_{vh}) \quad (1)$$

where *h* and *v* denote the horizontal and vertical orientations of the excitation and emission polarizer, respectively, and $g = I_{hv}/I_{hh}$ is an instrumental correction factor measured in a separate experiment and used to correct all subsequent experimental data. The temperature was maintained at 37 ^{o}C through the course of experiments.

Entrapment of CF and fluorescence assay of CF Release. The entrapment of 5(6)-Carboxyfluorescein (CF) in vesicles and its subsequent release was used to study liposome pemeability [11, 12]. After the chloroform was evaporated under vacuum, the DPPC film is redissolved in 50mM Tris-HCl buffer containing 50 mM CF. The suspension (1mM) is then subjected to sonication above 50 ^{0}C for 30 min. The liposomes loaded with CF were separated from the free dye by passing the mixture through a Sephadex G-50 column with 50 mM Tris-HCl, 100 mM NaCl as an elution buffer of. Several aliquots of the eluted fractions were collected and aliquot (100 μl) of one of the most CF concentrated fractions was taken and diluted to 2.9 ml with the elution buffer and the rate of CF leakage was measured at 37 ^{0}C by following the changes in emission due to CF as a function of time. The excitation and emission wavelengths used for this experiment were 495 and 520 nm, respectively. The CF release was measured relative to the total fluorescence intensity (I_f) obtained after disrupting the vesicular aggregate with 10% (w/v) Triton X-100 at the end of the experiment:

$$\% \text{ CF release} = (I_t - I_i)/(I_f - I_i) \times 100 \quad (2)$$

where I_i, I_t and I_f are the fluorescence emission values at time t =0, at time t and the final fluorescence on disruption of the vesicles, respectively.

3 RESULTS & DISCUSSION

Fluorescence Anisotropy. In order to elucidate the effect of different polymer systems on the fluidity of the fatty acyl chains in the interior of the bilayers, fluorescence anisotropy study is carried out. DPH is a very rigid and hydrophobic molecule and is extremely sensitive to the microviscosity of the environment since it is located in the interior segment of the vesicles, changes in the LDH anisotropy values indicate alteration in the motion of acyl chains of the phospholipids vesicles [10]. The anisotropy of DPH in the DPPC vesicles was found to be <*r*> =0.33 (Figure 2) at 37 ^{0}C, which is in agreement with others reported values [11, 13] since the transition temperature (T_c) of DPPC vesicles from gel to liquid crystalline state is known to be about 42 ^{0}C.

Figure 2 shows that the anisotropy value of DPH in DPPC vesicles remain constant (<*r*> = 0.33) with increasing concentration of either PDMA or PEO-*b*-PDMA. These results indicate that PDMA and PEO-*b*-PDMA have minimum interaction with the interior of the hydrophobic segment of the vesicles. At this buffer condition (50mM Tris-HCl with 100mM NaCl), majority of the amino group were charged and hence the interaction between the hydrophilic PDMA copolymer and the phospholipids vesicles were mainly restricted at the surface of the membrane. However, in the case of L92-*b*-PDMA, the anisotropy value of DPH decreases from 0.33 to 0.29 with increasing polymer concentration. Note that above the DPPC transition temperature (T_c) at 45 ^{0}C where the phospholipids were in the liquid crystalline state with higher fluidity, <*r*> = 0.20 was determined; therefore for

L92-*b*-PDMA, the decrease of <*r*> value represents 30% increase in the membrane fluidity. The decrease in <*r*> was strong indication of interaction between L92-*b*-PDMA and the hydrophobic fatty acid chains in the phospholipids vesicles.

b-PDMA. Note that pure DPPC vesicles are very resistant to the CF release at 37 ^{0}C due to the DPPC high gel-liquid crystalline transition temperature (~ 42 ^{0}C) compared to other phospholipids systems [11].

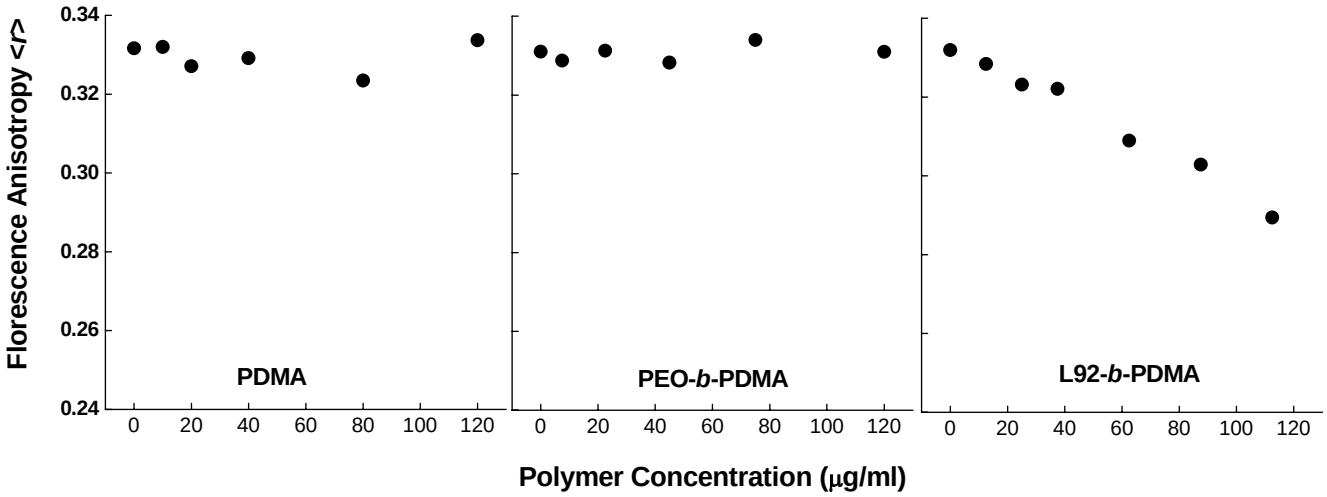


Figure 2: Effect of different polymer on the fluorescence anisotropy of DPH in phospholipids vesicles (1mM) in 100mM NaCl with Tris-HCl (50mM) buffer solution at pH 7.4 and 37 ^{0}C.

CF Release Assay. The influence of the different PDMA systems on the transmembrane permeation was investigated through the release of the self-quenching CF probe from DPPC phospholipids vesicles.

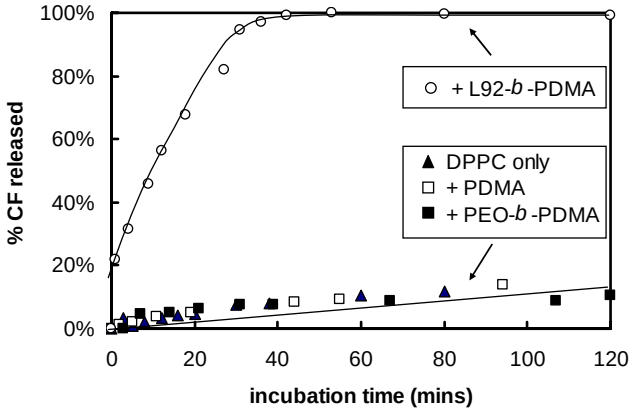


Figure 3: Time curve of the release of CF trapped in the phospholipids vesicles in the presence of polymers (20 μg/ml) at pH 7.4 and 37 ^{0}C.

Figure 3 shows that the CF release from DPPC vesicles varied dramatically in the presence of different polymethacrylate systems. The presence of PDMA or PEO-*b*-PDMA copolymers did not really affected the CF release, only about 10% of CF is released over a span of two hours with these polymers. On the other hand, nearly all CF were released after 40 mins of incubation in the presence of L92-

The results clearly demonstrated that the overall integrity of the vesicles remains intact in the presence of PDMA or PEO-*b*-PDMA, but the presence of L92-*b*-PDMA greatly affected the permeability of the vesicles causing the entrapped CF to leak out.

Under buffer condition of 50mM Tris-HCl with 100mM NaCl, majority of the amino group of the polymer were charged, PDMA and PEO-*b*-PDMA are hydrophilic, whereas the hydrophobic PPO segment in L92-*b*-PDMA resulted led to an amiphiphilic copolymers. The surface activity of L92-*b*-PDMA enhanced the interaction with the bilayer vesicles, which resulted in significant vesicles permeability. Other study on the effect of PEO and Pluronic polymers on CF release from phospholipids vesicles had shown similar results. Jonhsson *et al.* had showed that the presence of PEO alone (< 16% mol) would not cause any additional leakage of CF dye. He also compared the effect of several Pluronic polymers, Pluronic F127 which is relatively the most surface active, induced highest CF leakage [14].

Studies had shown that surface activity of the polymer or surfactant play a key role in the interaction with phospholipids membrane. Hydrophilic polymer have minimum interaction with the membrane as they were not able to partition into the bilayers [15]. Similarly, PDMA and PEO-*b*-PDMA being positive charged and hydrophilic probably only interact with the surface of the amphoteric DPPC vesicles that result in minimum alteration to the interior of the vesicles. On the other hand, amiphiphilic polymers are known to partition readily into the membrane

[15, 16], similarly in our case of L92-*b*-PDMA. In our experiment, the polymer concentration used (< 150 μg/ml) is well below the critical micelle concentration (cmc ~ 5 mg/ml) reported [3, 17], therefore the decrease in DPH anisotropy is primarily due to the incorporation of the polymer into the DPPC vesicle. The hydrophobic PPO segments of L92-*b*-PDMA interact with the interior fatty acyl chains of the phospholipids causing the bilayer vesicles to become more loosely packed that led to decrease in anisotropy and eventually led to leakage of the entrapped CF dye.

Recent studies have demonstrated the importance of the interaction between gene vector and cell membrane to cellular transfection efficacy – better transfection results can be achieved by coupling PDMA with other membrane associating molecules. Christiaens et al. demonstrated enhanced transfection efficacy by combining PDMA with a 16-residue water-soluble peptide that internalizes into cells through membrane translocation [5]. Funhoff et al. combining PDMA with a membrane-disrupting peptide derived from the influenza virus and achieved better transfection efficacy [6]. Whereas in this study, enhanced interaction between phospholipids L92-*b*-PDMA is also confirmed, which may be the reason behind the good transfection efficacy of L92-*b*-PDMA.

4 CONCLUSIONS

In this study, interaction between various cationic polymethacrylates (of different transfection efficiency) and phospholipids vesicles were evaluated. In general, the fluorescence anisotropy results agree with the CF release results. PDMA & PEO-*b*-PDMA which previously shown poor transfection results, have minimum interaction with the phospholipids vesicles, keeping the integrity of the DPPC vesicles intact. However, L92-*b*-PDMA that demonstrated enhanced transfection efficacy, showed significant interaction with the interior fatty acyl chains of the vesicles. The results of this study suggested that interaction between cell membrane and the delivery vectors play an important role in successful gene delivery.

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